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Inactivation of *Bacillus anthracis* in water by photocatalytic, photolytic and sonochemical treatment†

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Bacillus anthracis is one of the most dangerous and pathogenic bacterial species and its intrusion in aquatic environments is a serious threat to public health. The aim of the present study was to investigate inactivation rates of *B. anthracis* in water by means of photocatalytic (UVA/TiO_2), photolytic (UVC) and sonochemical treatment. The effect of various operating conditions such as bacterial concentration, TiO_2 loading, UV irradiation source, ultrasound power and treatment time was examined. The reference strain of *B. anthracis* proved to be highly resistant during photocatalytic and sonochemical treatment of aquatic samples, even in the presence of hydrogen peroxide solution, which is considered among the chemical disinfectants recommended for *B. anthracis* removal from aqueous suspensions. UVC irradiation was far more effective, as it achieved total inactivation in short treatment time (10 min) and at high initial concentrations (10^6 CFU mL^{-1}). The effectiveness of UVC irradiation is also reinforced by the fact that no photoreactivation occurred even after 72 h of exposure under sunlight after the end of the treatment. Furthermore, the virulence of residual cells was investigated, targeting two genes carried in the plasmids pXO1 and pXO2, respectively, which are required for a fully virulent type. Interestingly, the plasmid pXO2 seems to be lost from the host after photocatalytic and photolytic disinfection, resulting in apathogenic residual strains contained in the treated sample. Overall, our results highlight the importance of *B. anthracis* efficient inactivation in water, as it shows considerable resistance towards effective and reliable disinfection techniques.

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1. Introduction

Bacillus anthracis is enlisted among the most dangerous and pathogenic bacterial species, capable of causing serious and often fatal infection to livestock and humans. According to the Center for Disease Control and Prevention (CDC) it is a category A agent with potential for a major public health impact.[1] It is a spore-forming Gram-positive bacterium that can be easily disseminated or transmitted from person to person and cause the acute mammalian disease anthrax. Its virulence is associated with two plasmids pXO1 and pXO2, which are potentially contained in the cell encoding toxin and antiphagocytic capsule genes, respectively.[2,3] Either or both of these plasmids may be lost from the host under stressful conditions induced in nature or during laboratory manipulation.[4]

Whereas *B. anthracis* is well known as a biological warfare agent intended for aerosol application, it has also been identified as a water threat, as it may inflict severe symptoms when ingested.[5] Despite the controversy concerning the long-term viability of this strain in water, reports have shown that its spores may be stable in this environment for 2 years, while their survival is feasible even in seawater or distilled water.[6] Taking into account that a water intrusion event referred to this bacterium cannot be easily prevented, it is imperative to explore effective and reliable disinfection techniques, which would eventually inactivate such high priority agents, providing all required actions for public health preparedness.

However, there is limited experimental evidence regarding *B. anthracis* inactivation and the mechanisms by which spores resist the lethal effects of various disinfection treatments in water systems. Most disinfection approaches have been evaluated using *B. anthracis* surrogates, such as *Bacillus subtilis, Bacillus globigii* and *Bacillus cereus*.[7–10] Recommendations by the Environmental Protection Agency (EPA) include mainly

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chemical disinfectants, among which are sodium hypochlorite and liquid chlorine dioxide.[6] According to previous studies, viable spore counts may be reduced more than two logs within 2 h when using chlorine dioxide, while free chlorine achieves minimal spore inactivation (2 log reduction after 7 h at 1 mg L^{-1} free chlorine) and even higher concentrations are required when spores are embedded in biofilms.[5,7] Although these chemicals have exhibited certain efficacy against the *Bacillus* spores, considerable health hazards and destructive properties are associated with them.[10] Therefore, there is a quest for alternative disinfection methods, especially when human contact occurs or sensitive equipment and materials are present.

Given that an efficient and relatively safe biocide should have high oxidation potential, advanced oxidation processes (AOPs) have been recognised as an emerging group of techniques, highly effective for inactivating pathogenic bacteria in aqueous samples.[11,12] Among them photocatalysis (UVA/TiO_2) and UVC irradiation have been extensively investigated regarding water and wastewater disinfection with respect to common faecal bacterial indicators, but considerably less experimental evidence is available regarding *B. anthracis* inactivation.[13] The oxidative stress imposed by AOPs and the hazardous effect of UVC light produced in the DNA and in the outer microbial membrane cell make these methods reliable non-reagent techniques for water/wastewater disinfection. Another promising method in terms of pathogen destruction in suspensions is ultrasound irradiation, which has already been recognized for its advantages in water treatment.[14] High intensity ultrasound is detrimental to microorganisms in aqueous matrices as it induces cell lysis or increases the permeability of their membranes, thus spilling their contents.[15]

In the present study we focused on the evaluation of selected water/wastewater treatment methodologies in terms of *B. anthracis* inactivation in aqueous matrices. Specifically, photocatalytic (UVA/TiO_2), photolytic (UVC) and sonochemical treatments were tested regarding their disinfection efficiency, examining the effect of various operating conditions such as bacterial concentration, TiO_2 loading, UV irradiation source, ultrasound power and treatment time. Furthermore, disinfection durability experiments were carried out. Finally, apart from viable counts and given the pathogenicity of the bacterial strain, we attempted to assess the virulence of residual cells in each case, targeting and quantifying two genes carried in the plasmids pXO1 and pXO2, respectively.

2. Experimental

2.1. Bacterial strain

The reference bacterial strain used in the present study was *B. anthracis* NCTC 10340 (HPA Culture Collections). Bacterial suspensions were prepared in a sterile 0.8% (w/v) NaCl aqueous solution and cell concentration was estimated measuring the optical density at 600 nm (Shimadzu UV1240 spectrophotometer). Bacterial suspensions in the range of 10^3–10^6 CFU mL^{-1} were used as samples for disinfection experiments. All experiments were carried out in a biosafety level 3 laboratory.

2.2. Photocatalytic/photolytic experiments

Disinfection experiments were conducted in an immersion well, batch type, laboratory scale photoreactor (Ace Glass, Vineland, NJ, USA) with a total volume of 300 mL and an irradiated surface of 330 cm^2. During experimental runs 300 mL of the 0.8% (w/v) NaCl bacterial suspension was introduced into the reaction vessel and the appropriate amount of catalyst (TiO_2), when required, was added to achieve the desirable catalyst loading. The catalyst used in this study was a commercially available TiO_2 (Aeroxide P-25) powder supplied by Evonik Industries. Its physicochemical characteristics are anatase : rutile (75 : 25), particle size of 21 nm and its BET area is 50 m^2 g^{-1}. Two different TiO_2 loadings were tested for the inactivation of *B. anthracis* during photocatalytic experiments, namely 1 g L^{-1} and 1.5 g L^{-1}. These specific loadings were chosen taking into account that our reference bacterial strain is a Gram-positive microorganism, which possesses a complex wall structure and is highly resistant to disinfection techniques. Similar catalyst concentrations have been proved suitable for inactivation of other Gram-positive microorganisms in various aqueous matrices.[12]

UVA irradiation was provided by a 9 W lamp (Radium Ralutec, 9W/78, 350–400 nm). UVC irradiation was provided by an 11 W lamp (Philips, TUV, 11 W, PL-S). The suspension was magnetically stirred for 40 min in the dark to ensure complete equilibration of adsorption/desorption of *B. anthracis* bacteria onto the catalyst surface and subsequently the UV lamp was turned on. The reaction mixture was continuously stirred and the temperature was maintained at 25 ± 1 °C with a temperature control unit. The external reaction vessel was covered with aluminum foil to reflect irradiation exerted on the outer wall of the reaction vessel. Experiments were carried out in triplicate to check the repeatability of the process. Also, UVA/TiO_2 photocatalytic treatment was performed under the same conditions as mentioned previously, with 1.5 g L^{-1} TiO_2 and 20 mg L^{-1} H_2O_2.

Disinfection durability experiments were performed to determine the efficiency of the UV irradiation treatment. In order to evaluate the bacterial photoregeneration, after the disinfection treatment, 100 mL of the final effluent were kept in the dark and another 100 mL were irradiated by natural sunlight under continuous stirring for 3 days. After this period of time the final sample was analyzed in terms of *B. anthracis* viability.

2.3. Sonochemical experiments

Sonochemical experiments were performed using an Ultrason 250 (LabPlant, Huddersfield, UK) horn-type sonicator capable of operating either continuously or in pulse mode at a fixed frequency of 80 kHz and a variable power output up to 150 W (nominal) was used. Reactions took place in a cylindrical pyrex cell in a volume of 300 mL under continuous stirring and at a constant temperature of 25 ± 1 °C. Ultrasound irradiation was

emitted through a 1 cm diameter tip which was positioned in the middle of the cell with the tip 4.2 cm from the bottom. The power density emitted in the liquid was in the range 18–46 W L^{-1} as determined calorimetrically. Experimental runs were also performed in the presence of H_2O_2, which was provided by Merck in the form of 35% (w/w) solution. The concentration of H_2O_2 during sonochemical treatment was 20 mg L^{-1} and was monitored using Merck peroxide test strips (and so was in photocatalytic experiments).

2.4. *B. anthracis* viable counts

During each treatment bacterial viability was determined applying the serial dilution streak plate agar technique. In each case and at various time points, 3 mL from the reaction vessel were withdrawn and were serially diluted in a sterile 0.8% (w/v) NaCl aqueous solution. Aliquots of 200 μL of each dilution were streaked onto Nutrient Agar plates (OXOID), followed by incubation at 37 °C for 24 h. The minimum detectable number of bacteria in these experiments was 1 CFU mL^{-1}, a reflection of the fact that 1000 μL of each dilution (5 × 200 μL) were streaked on agar plates.

2.5. DNA isolation and real-time PCR method

DNA was extracted from treated *B. anthracis* colonies using QIAamp DNA mini kits (Qiagen) supplemented with lysozyme lysis buffer (100 mM NaCl, 500 mM Tris [pH 8], lysozyme 10 mg mL^{-1}). The quantity and purity of all DNA samples were determined measuring their absorbance value at 260 nm and estimating the ratio of absorbance values at 260 nm and 280 nm, respectively.

The primers used for PCR detection of *B. anthracis* are shown in Table 1. Overall, two plasmid genes (lef gene in plasmid pXO1 and capC gene in plasmid pXO2) were targeted.[4,16] The SYBR green method was applied using the StepOne Plus System (Applied Biosystems Inc., Foster City, CA, USA). Triplicate PCR reactions were carried out with a KAPA™-SYBR® FAST Real-time PCR Kit (Kapa Biosystems) to a final volume of 20 μL. The mixed PCR solution contained a 2XPCR master mix, 0.5 μM of each primer and 2 μL of crude cell lysate, all diluted to the final volume of the reaction mixture with DNase/RNase free water. Amplification was accomplished by initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 58 °C for 30 s, and extension at 72 °C for 10 s. Melt curve analysis was performed by slowly heating the PCR mixtures from 60 to 95 °C (1 °C per cycle of 10 s) with simultaneous measurements of the SYBR Green signal intensities.

To determine the detection sensitivity of the real-time PCR assay, a series of 10-fold diluted pure culture genomic and plasmid DNA from the reference strain was tested for real-time PCR amplification and cycle threshold (C_T). The generated standard curves were used to estimate the amount of DNA detected in each treated sample. Also, they reflect the efficacy of the PCR assay as a quantification method, considering the values of linear correlation coefficient and slope, which were 0.97 and −3.30 for lef gene and 0.98 and −3.10 for capC gene.[17]

3. Results and discussion

3.1. Photocatalytic treatment

Aqueous suspensions of *B. anthracis* were subject to photocatalysis in order to assess the synergistic effect of UVA light treatment and catalyst TiO_2 loading on the inactivation of this pathogenic bacterial strain. Considering that concentration of the catalyst in slurry photocatalytic treatment strongly affects the overall process, two different TiO_2 loadings (1 g L^{-1} and 1.5 g L^{-1}) were tested and colony counts were recorded at various intervals of time until 60 min (Fig. 1). According to our results, increasing the catalyst loading improved inactivation rates of *B. anthracis*, as a 4-log reduction of bacterial population was recorded within 1 h when 1.5 g L^{-1} TiO_2 were used. However, total bacteria killing was not achieved in either case highlighting, to a certain extent, the virulence and the resistance of this specific bacterial strain during water disinfection

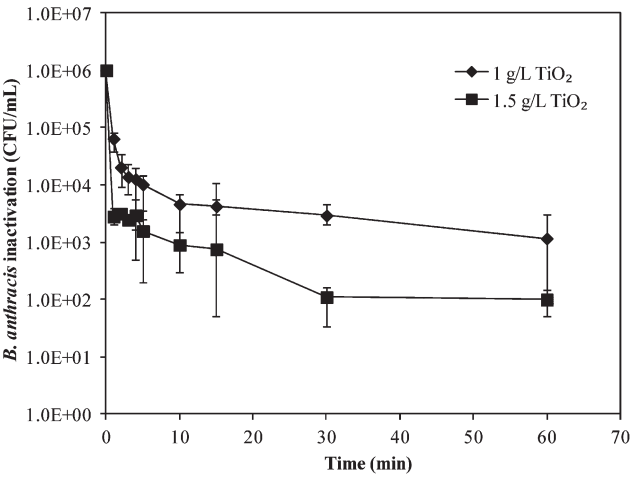

Fig. 1 *B. anthracis* inactivation in a 0.8% (w/v) NaCl aqueous solution for two TiO_2 loadings under UVA irradiation.

Table 1 Primers for *B. anthracis* real-time PCR

Loci	Primers	Sequence (5′ → 3′)	Product (bp)	T_m (°C)
Lef (pXO1)	Lef4 F	TGAACCCGTACTTGTAATCCAATC	475	67.6
	Lef4 R	ATCGCTCCAGTGTTGATAGTGCT		68
CapC (pXO2)	Cap29 F	GTTGTACCTGGTTATTTAGCACTC	318	62.7
	Cap29 R	ACCACTTAACAAAATTGTAGTTCC		62.3

treatment. Conversely, in a relevant study where various quantities of nanosized titania were used but with lower initial population (1900 CFU mL^{-1}), total inactivation of vegetative cells did occur within 1 h.[18] The inherent resistance of *B. anthracis* in the present study may be partially attributed to the high cellular starting quantity combined with the complex wall structure possessed by this bacterium. Higher resistance profiles have been recorded during spore treatment.[19] Spores have complex nature and chemical composition, demonstrating enhanced resistance towards disinfection techniques.[5] Nevertheless, titania nanomaterials and UVA or even sunlight have the potential of efficiently inactivating spores of *B. anthracis*, possibly because of cellular fluids leakage, which follows spore damage.[19]

The bactericidal effect of TiO_2, which involves loss of membrane integrity, is well known but it is associated with the initial bacterial density in each case. In this sense, experimental runs were performed with two different initial concentrations (10^6 and 10^4 CFU mL^{-1}) and the viable count results are illustrated in Fig. 2a. Applying these two bacterial densities, the overall cell reduction which was achieved within 90 min of irradiation was 4 and less than 2 orders of magnitude, respectively. Although the highest catalyst loading was employed (1.5 g L^{-1}) total disinfection did not occur even when 10^4 CFU mL^{-1} were contained in the suspension. In most photocatalytic experiments an increase in bacterial concentration leads to a decrease in the inactivation rate.[11,12] However, in our case, although initial bacterial concentrations had a difference of 2 orders of magnitude, the density of the residual cells after treatment was approximately the same. Furthermore, in approximately 40 min residual *B. anthracis* cells reached a plateau, beyond which no significant population decrease was recorded, implying a threshold under the applied experimental conditions for maximum inactivation and degradation of the used bacterial strain. It should be pointed out though that at the conditions in question, there is lack of a considerable initial lag shoulder, which is usually observed when specific chemical disinfectants are used for *B. anthracis* inactivation, like chlorine and chlorine dioxide.[7] Therefore UVA/TiO_2 photocatalysis seems to induce direct cellular destruction in aqueous matrices.

In an attempt to evaluate the consistency and virulence of residual *Bacillus* cells and taking into account that cultivability is not synonymous to viability for bacteria,[13] SYBR green real-time PCR was performed, targeting two genes included in plasmids pXO1 and pXO2 (Table 1). One approach to distinguish between pathogenic and apathogenic *B. anthracis* strains is to investigate the presence of those two plasmids, which are both required for a fully virulent type.[4,16,20] pXO1 and pXO2 carry genes for toxin synthesis (pag, cya, and lef) and capsule synthesis (capB, capC, capA, and capD), respectively.[16] Real-time PCR provides the potential for estimating the number of bacterial cells contained in various samples through the quantification of the abovementioned genes. According to the literature and considering that we used a reference strain provided by HPA (Health Protection Agency), each cell contains

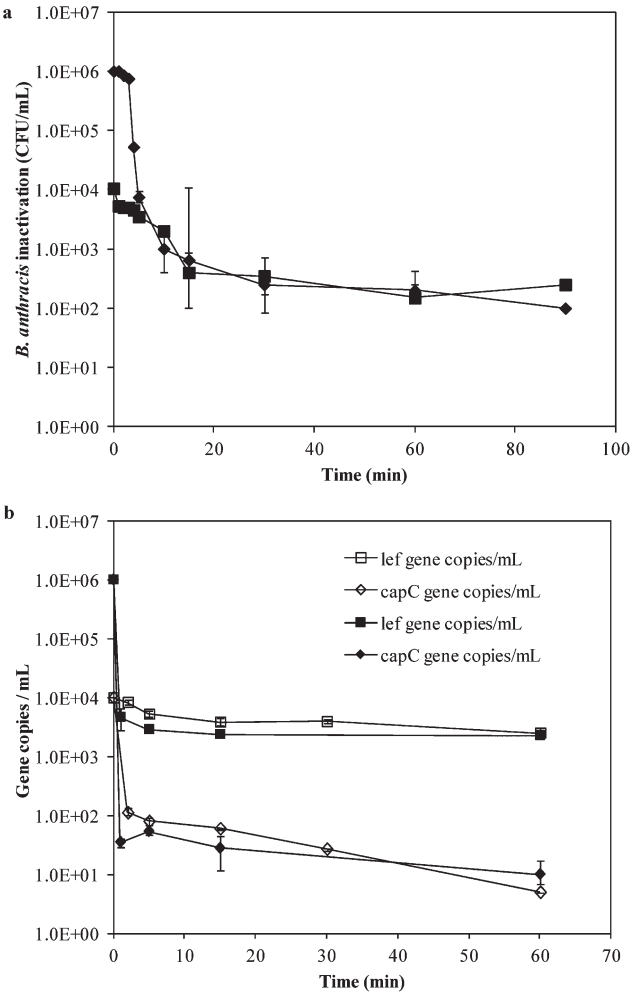

Fig. 2 *B. anthracis* inactivation in a 0.8% (w/v) NaCl aqueous solution during treatment by UVA/TiO_2, [TiO_2] = 1.5 g L^{-1}, assessed by (a) the culture technique and (b) real-time PCR [◊ & □: lef and capC gene copies L^{-1}, corresponding to an initial concentration of 10^4 CFU mL^{-1}; ◆ & ■: lef and capC gene copies L^{-1}, corresponding to an initial concentration of 10^6 CFU mL^{-1}].

one copy of lef and capC genes. Gene copies numbers may be correlated to cell numbers recorded by culture techniques. However, this comparison is not quite consistent, since PCR has higher detection sensitivity than the agar plate method, taking into account that a considerable number of bacterial cells lose their ability to grow on solid culture media under stressed conditions, like those imposed during disinfection.

In the present study, quantification of the genes lef (plasmid pXO1) and capC (pXO2) was performed after UVA/TiO_2 treatment and the obtained results are shown in Fig. 2b. During photocatalysis and testing two initial *B. anthracis* concentrations, there was a significant decrease of capC gene copies in the residual cells, which was over 5 orders of magnitude, while lef gene copies remained in high densities even after 1 h of disinfection. Decrease profiles of both genes were similar for the two starting quantities tested. Loss of capC gene during treatment implies the inability of the bacteria to form the necessary capsule, which in turn confers resistance

to phagocytosis.[16] In the present case, the residual cells, which were recorded after prolonged photocatalysis, seem to have lost plasmid pXO2, leading to apathogenic bacteria remained after treatment. Generally, the plasmids may be lost from the host under extreme environmental conditions, like those induced by a UVA/TiO_2 process. In our case, the loss of plasmid pXO2 may be attributed to photocatalytic treatment, since several laboratory tests were performed prior to UVA/TiO_2 treatment (*i.e.* several recultures of the bacterial strain with subsequent PCR reaction and amplification of lef and capC genes), without recording any loss of the two plasmids. The effectiveness of UV irradiation regarding microbial inactivation is attributed to the fact that DNA molecules absorb UV photons between 200 and 300 nm, with a peak absorption at 265 nm.[21] The extremely reactive $OH^{\cdot}$, for which no defence exists, is able to damage DNA, leading to misreading of the genetic code and finally cell death.[22]

3.2. Photocatalytic treatment with hydrogen peroxide solution

Photocatalytic treatment was performed under the same conditions as mentioned previously, with 1.5 g L^{-1} TiO_2 and 20 mg L^{-1} H_2O_2. Disinfection efficiency of the process was slightly enhanced but even in the presence of H_2O_2 100% bacterial killing did not occur (Fig. 3). Testing two different initial concentrations, 10^4 CFU mL^{-1} and 10^6 CFU mL^{-1}, the overall decrease was approximately of 3 and 5.5 orders of magnitude, respectively. As the Environmental Protection Agency (EPA) requires, a biocide should demonstrate a 6-log inactivation in order to be registered as pesticide. Yet, in the case of *B. anthracis* in a water system, a potential release would result in a final concentration much lower than 10^6 spores mL^{-1}, especially after dilution from the point of discharge. In this sense, hydrogen peroxide could achieve satisfactory inactivation rates, particularly in relation to UVA/TiO_2 photocatalytic treatment. Hydrogen peroxide is generally recommended as a chemical disinfectant against *B. anthracis*, as it has exhibited efficacy for the inactivation of this strain.[6,23] Available experimental evidence is very little and most of the studies deal with spores of *B. anthracis*. According to Raber and Burklund, a solution of 0.5% was almost 100% effective at a spore concentration of 10^2 mL^{-1} and a contact time of 10 min.[6]

Screening real-time PCR results, it is noteworthy that decrease profiles of the two plasmid genes were enhanced in the presence of hydrogen peroxide compared with those obtained applying UVA/TiO_2 (Fig. 3b). Gene copies of capC were almost not detectable after 1 h of treatment of aqueous suspensions inoculated with 10^4 CFU mL^{-1} and 10^6 CFU mL^{-1}. Loss of the corresponding plasmid implies that residual cells do not retain their pathogenicity as they are no longer recognised as fully virulent types. Interpretation of real-time PCR results requires extra caution, in terms of using DNA as a reliable parameter to quantify viable organisms exposed to stressed conditions. If properly designed, PCR could be a substantial and reliable molecular tool for the quantification of microbial DNA in environmental samples. Proper primer

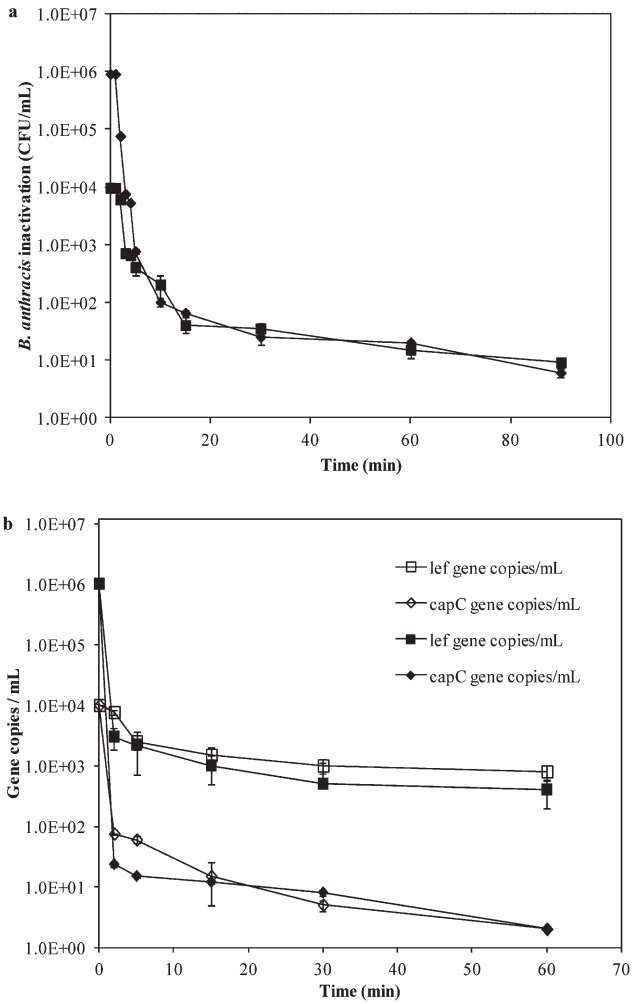

Fig. 3 *B. anthracis* inactivation in a 0.8% (w/v) NaCl aqueous solution during treatment by UVA/TiO_2, [TiO_2] = 1.5 g L^{-1} with 20 mg L^{-1} H_2O_2, assessed by (a) the culture technique and (b) real-time PCR [◊ & □: lef and capC gene copies L^{-1}, corresponding to an initial concentration of 10^4 CFU mL^{-1}; ◆ & ■: lef and capC gene copies L^{-1}, corresponding to an initial concentration of 10^6 CFU mL^{-1}].

design and suitable product size are prerequisites for valuable results, considering that the longer the targeted DNA fragment is, the more UV-induced DNA lesions inhibit the PCR.[24,25] In this study the amplicon sizes for the two targeted plasmid genes were 475 bp and 318 bp for lef and capC gene, respectively, ensuring accurate molecular biology reduction rates. From a more skeptical point of view, loss of plasmid pXO2 could be attributed to laboratory manipulation. Nonetheless, the repeatability of specific plasmid loss during all experimental runs, combined with extremely careful handling of bacteria, suggests that pXO2 was decayed as a consequence of photocatalytic treatment.

3.3. Photolytic treatment

Experimental runs were conducted in order to achieve better inactivation rates of *B. anthracis.* Photolytic experiments were carried out applying UVC irradiation and using an inoculated

0.8% (w/v) NaCl aqueous solution. Disinfection rates assessed by viable counts and real-time PCR are demonstrated in Fig. 4. UVC irradiation proved to be far more effective than UVA/TiO_2 photocatalytic treatment as 100% bacteria killing occurred within the first 10 min of the treatment, even though the starting quantity of cells was 10^6 CFU mL^{-1}. UVC irradiation is considered as one of the most powerful stressing agents, which induces DNA lesions and cell death in short time periods and has already been investigated as disinfection means. The vast majority of published experimental data deal with common faecal bacterial indicators, whereas more pathogenic and virulent species like *B. anthracis* remain unexplored. Interestingly and despite the inherent disinfection resistance of this bacterium against many applications, like chemical treatment or UVA/TiO_2,[18,19,26,27] UVC seems quite promising for water treatment, taking into account that in the present case concentration of bacteria after the process reached a non-detectable level.

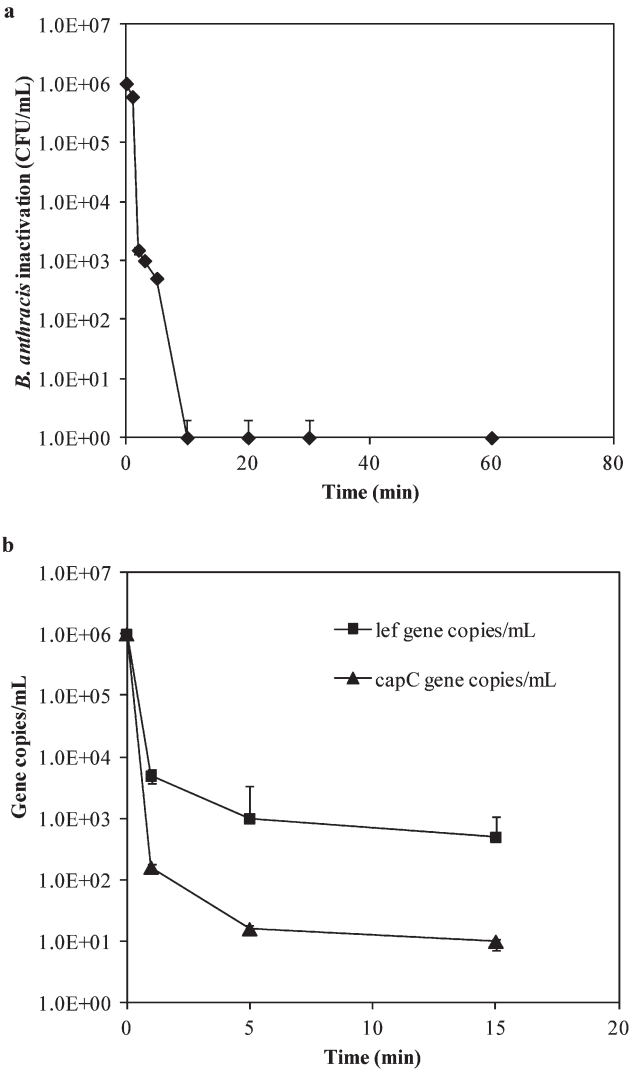

Fig. 4 *B. anthracis* inactivation in a 0.8% (w/v) NaCl aqueous solution during treatment by UVC assessed by (a) the culture technique and (b) real-time PCR.

Regarding real-time PCR, results were similar to those obtained after application of photocatalysis. CapC gene copies were decreased over 5 orders of magnitude in the first 15 min of irradiation, whereas lef gene copies remained at high levels. The discrepancy between PCR and viable count results is probably attributed either to loss of culturability of the remained cells or to sensitivity of the DNA based technique. Conventional culture is the golden standard for measuring microbial inactivation rates, but under certain conditions PCR may be quite useful and supplementary providing further information.[28] The presence and quantification of plasmids pXO1 and pXO2, and therefore the assessment of *B. anthracis* virulence could not be performed otherwise. However, one of the main drawbacks of real-time PCR should be under consideration and that is the sensitivity and detection limit when it comes to environmental samples, like water and aqueous matrices. The environmental limit of detection for tap water is reported as 10 CFU 10 L^{-1} using the cultivation methods, while for PCR based methods, the environmental limit of detection is decreased to 534 CFU L^{-1}.[1] Usually, cultivation is employed to determine the viability of the organisms in the sample, and PCR for confirmation tests, such as microbial virulence, which was our case.

3.4. Durability of photocatalytic and photolytic treatment

When photolytic and photocatalytic treatments are applied, it is important to verify the disinfection durability, as generated oxidative species have short half-life and microbial reactivation may occur. Under natural sunlight, an enzymatic reaction which is called photoreactivation, may occur and lead to the proliferation of pathogens, reducing UV disinfection efficiency.

In the present study disinfection durability experiments were carried out in the dark, as well as under natural sunlight irradiation, so as to determine the efficiency of the photodegradation of the microorganisms. Reactivation occurred only in the case of UVA/TiO_2 photocatalytic treatment and when the reaction sample was maintained under solar light, indicating that the oxidative species developed on the titania surface did not cause severe damage to the cells. Bacterial cells were reactivated after 48 h of exposure to sunlight and in the range of 20–30% of initial concentration. The same percentage corresponds to the plasmids genes, which were detected after 48 h of exposure to sunlight. Conversely, after UVC irradiation no regeneration of cells and no plasmid genes detection were recorded, indicating unrecoverable and severe DNA damage. Based upon these results, photolytic treatment applying UVC irradiation is superior to UVA/TiO_2 in terms of permanent inactivation of *B. anthracis* in aquatic samples.

3.5. Sonochemical treatment

Experimental runs were also carried out applying sonochemical treatment with 20 mg L^{-1} hydrogen peroxide added for the inactivation of the strain in a 0.8% (w/v) NaCl aqueous solution. According to our results, increasing the ultrasound power resulted in a slight enhancement of *B. anthracis* inactivation (Fig. 5). Despite the presence of H_2O_2 and the relatively low

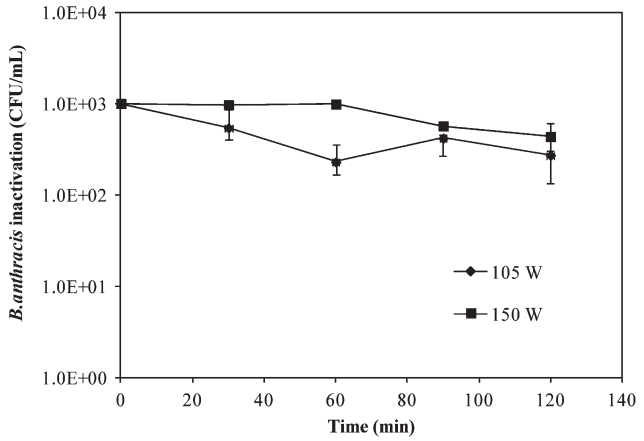

Fig. 5 *B. anthracis* inactivation in a 0.8% (w/v) NaCl aqueous solution during sonochemical treatment with 20 mg L^{-1} H_2O_2.

cell density (10^3 CFU mL^{-1}), the overall process was not quite effective. Complete inactivation did not occur even after 120 min of treatment. Therefore, sonochemical treatment, at least under the specified conditions, is not suitable for *B. anthracis* inactivation in water. Nevertheless, high intensity ultrasound is commonly used to remove bacterial cells, especially from surfaces. However, even at very high power levels, not all of the bacteria are removed (*e.g. Proteus mirabilis*).[15] Low inactivation rates recorded in the present study are probably due to the complex structure of *B. anthracis* cells.

4. Conclusions

– On the whole, this study highlights different aspects concerning inactivation of *B. anthracis* in aqueous solutions by means of photocatalytic, photolytic and sonochemical treatments. The reference strain *B. anthracis* showed inherent resistance towards UVA/TiO_2 and sonochemical processes, even in the presence of hydrogen peroxide solution. Conversely, UVC irradiation was far more effective for complete inactivation of the bacterium under the specified experimental conditions.

– Based upon our results, the potential of photoreactivation of residual *B. anthracis* after photocatalysis and photolysis should be under consideration, since it may occur, especially in the case of UVA/TiO_2 photocatalytic treatment.

– The virulence of *B. anthracis* in water, which is due to the carried plasmids pXO1 and pXO2, raises many concerns for public health protection. During UVA/TiO_2 and UVC treatments and under the present experimental conditions, it seems that pXO2 may be lost from residual bacterial cells, resulting in apathogenic strains.

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